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Assistant Commissioner for Patents  
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Sir:

The following utility patent application is enclosed for filing:

Applicant(s): Donald Frank Cameron and Helena Selawry

Executed on: Unexecuted

Title of Invention: METHODS OF TREATING DISEASE USING SERTOLI  
CELLS AND ALLOGRAFTS OR XENOGRAFTS

Pages of Specification 57

Sheets of Drawings 12

## PATENT APPLICATION FEE VALUE

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Total					1,934.00
50% Reduction for Independent Inventor, Nonprofit Organization or Small Business Concern (a verified statement as to the applicant's status is attached)					-
Total Filing Fee					\$ 1,934.00

This application claims priority under Title 35, United States Code, §120 to the application entitled "METHODS OF TREATING DISEASE USING SERTOLI CELLS AND ALLOGRAFTS OR XENOGRAFTS" of Serial No. \_\_\_\_\_, filed on \_\_\_\_\_, which is a continuation-in-part application of Serial No. 08/221,695, filed on April 13, 1994.

Please charge the required fee to Pennie & Edmonds Deposit Account No. 16-1150. A copy of this sheet is enclosed.

Respectfully submitted,

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Enclosure

## EXPRESS MAIL CERTIFICATION

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METHODS OF TREATING DISEASE USING  
SERTOLI CELLS AND ALLOGRAFTS OR XENOGRAFTS

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This invention was made with United States government support under grant DK42421 awarded by the National Institutes of Health. The United States Government may have certain rights in the invention.

FIELD OF THE INVENTION

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Transplants of healthy organs or cells into a patient suffering from a disease are often rejected by the body due to an immune response initiated in response to the foreign tissue or cells. The present invention provides a method of cellular transplantation in which an immunologically privileged site is created, thus alleviating the rejection associated with conventional transplantation therapy. Specifically, the present invention describes a method of treating a disease that results from a deficiency of a biological factor which comprises administering to a mammal Sertoli cells and cells that produce the biological factor. In particular, the present invention describes a method of treating diabetes mellitus by transplanting pancreatic islet of Langerhans cells in conjunction with Sertoli cells to create an immunologically privileged site. A method of creating an immunologically privileged site in a mammal for cellular transplants is further described by the present invention. A pharmaceutical composition comprising Sertoli cells and cells that produce a biological factor is also provided.

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**BACKGROUND OF THE INVENTION**

5 Certain chronic diseases destroy the functional cells in affected organs. Mammals with such diseases are often unable to produce proteins or hormones necessary to maintain homeostasis and usually require numerous exogenous substances to survive. Transplanting healthy organs or cells into a mammal suffering from such a disease may be necessary to save the mammal's life. This type of therapy is generally regarded as a last alternative to curing an otherwise fatal condition. Such transplants, however, are often rejected by the body due to an immune response initiated in response to the foreign tissue or cells. Presently, the only recourse to combat this immune response is to administer chronic nonspecific immunosuppression agents. Unfortunately, this only trades the complications of one chronic disease with other complications caused by the immunosuppression agent.

20 One disease which scientists have attempted to treat with organ and/or cellular transplants but have had very limited success is diabetes mellitus. Diabetes mellitus is a prevalent degenerative disease in mammals. It is characterized by a relative or complete lack of insulin secretion by the beta cells within the islets of Langerhans of the pancreas or by defective insulin receptors.

25 This insulin deficiency prevents normal regulation of blood glucose levels and often leads to hyperglycemia and ketoacidosis. When administered to a mammal, insulin promotes glucose utilization, protein

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1 synthesis, formation and storage of neutral lipids and  
the growth of certain cell types.

5 In the United States alone there are  
approximately 13 million diabetics. Of these, 2.6  
million are insulin dependent diabetics. Drug & Market  
Dev., 4:210 (1994). Healthcare analysts estimate that  
diabetes costs \$92 billion a year resulting from medical  
costs and lost productivity.

10 The various forms of diabetes have been  
organized into a series of categories developed by the  
National Diabetes Data Group of the National Institutes  
of Health. Type I diabetes in this classification  
15 scheme includes patients dependent upon insulin to  
prevent ketosis. This group of diabetics was previously  
called juvenile-onset diabetes, brittle diabetes or  
ketosis-prone diabetes. Type I diabetes is caused by an  
autoimmune reaction that causes complete destruction of  
beta cells.

20 Type II diabetes is classified as adult-onset  
diabetics. The diabetic patient may or may not be  
insulin dependant. Type II diabetes can be caused by a  
number of factors. For most mammals with Type II  
diabetes, the beta islet cells are defective in the  
25 secretion of insulin.

30 There are many therapies currently used to  
treat diabetes, however, each has its limitations. The  
major problem confronting most patients with diabetes  
mellitus is that currently available therapies fail to  
prevent the complications of the disease process. The  
most common method of treating Type I diabetes in  
mammals is providing an endogenous source of insulin

1 such as porcine, bovine or human insulin. Insulin  
injection therapy prevents severe hyperglycemia and  
5 ketoacidosis, but does not completely normalize blood  
glucose levels. This treatment further fails to prevent  
the complications of the disease process, including  
premature vascular deterioration. Premature vascular  
deterioration is the leading cause of morbidity among  
diabetic patients. Furthermore, complications resulting  
10 from long-term diabetes include renal failure, retinal  
deterioration, angina pectoris, arteriosclerosis,  
myocardial infarction and peripheral neuropathy.

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15 A second method of treating diabetes is by  
transplanting the pancreas in conjunction with the  
administration of chronic nonspecific immunosuppression  
agents. This treatment is usually given to an  
individual who has advanced diabetes, such as an  
individual with kidney failure. Whole pancreas  
transplantation can be successfully done with a 75% one-  
20 year survival rate, but surgical transplantation of the  
pancreas is very difficult. Furthermore, since the  
entire organ must be donated, the only practicable  
source is a deceased donor. In addition, when  
cyclosporine, the most common immunosuppressive drug  
25 used for organ transplants, is administered in a dosage  
necessary to suppress the immune response, the drug  
inhibits pancreatic cell function. Furthermore, the  
steroids that are often administered with an organ  
transplant often cause the patient to become diabetic.

30 A third treatment involves transplanting islet  
of Langerhans cells into the diabetic patient. However,  
islet transplantation has been generally unsuccessful due

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to the aggressive immune rejection of islet grafts.  
(Gray, 1991, Immunology Letters 29:153; Jung et al.,  
1990, Seminars in Surgical Oncology 6:122). In

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particular, successful transplantation of isolated  
pancreatic islet cells has been very difficult to  
achieve due to the chronic administration of  
immunosuppressive drugs required to prevent organ  
rejection of the cells following transplantation. These  
10 dosages of immunosuppressive drugs can cause increased  
susceptibility to infection, hypertension, renal failure  
and tumor growth. Furthermore, unlike most organ  
transplants, islet cells must grow their own blood  
supply following implantation in the host in order for  
15 the cells to survive. Conventional transplantation  
techniques do not provide the necessary factors to  
stimulate the production of new blood vessels.

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The present invention alleviates many of the  
problems associated with the current therapies for  
20 chronic diseases that destroy the functional cells of  
vital organs. In particular, the present invention  
solves the problems associated with the conventional  
therapies for diabetes mellitus, by providing a method  
of transplanting pancreatic islets cells into a diabetic  
25 mammal, whereby the cellular transplants produce insulin  
in the diabetic mammal. The present inventor has  
previously demonstrated extended functional survival of  
islet cells allografts and xenografts in the testis.  
(Selawry et al., 1989, Diabetes 38:220.) It has been  
30 surprisingly discovered in accordance with the present  
invention that an immunologically privileged site can be  
created in a mammal by transplanting Sertoli cells to a

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nontesticular site in a mammal. The newly created immunologically privileged site allows the transplantation and survival of cells that produce biological factors useful in the treatment of diseases, especially diabetes.

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#### SUMMARY OF THE INVENTION

The present invention relates to a method of treating a disease that results from a deficiency of a biological factor in a mammal which comprises administering Sertoli cells and cells that produce the biological factor. In a preferred embodiment, the biological factor is a hormone.

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In a more preferred embodiment, the disease is diabetes mellitus, the factor producing cells are pancreatic islet cells and the factor is insulin.

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In yet another embodiment the cells that produce the biological factors are cells that have been genetically engineered, for example by transformation with a nucleic acid that expresses the biological factor.

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The present invention further relates to a method of treating diabetes mellitus in a mammal comprising administering pancreatic islet cells and Sertoli cells. In a preferred embodiment the Sertoli cells and islet cells are administered by transplantation.

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Another aspect of this invention is directed to a method of creating an immunologically privileged site in a mammal.

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1 Yet another embodiment of the present  
invention provides a pharmaceutical composition  
comprising Sertoli cells and cells that produce a  
5 biological factor. In a preferred embodiment the  
pharmaceutical composition comprises Sertoli cells and  
pancreatic islet cells and a pharmaceutically acceptable  
carrier.

10 The present invention further provides a  
compartmentalized kit containing Sertoli cells and cells  
that produce a biological factor. An article of  
manufacture comprising a packaging material and Sertoli  
cells contained within the packaging is also provided.

15 **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows the glucose responses to oral  
sustacal tolerance tests done on the monkey "Lucky" at  
intervals before pancreatectomy (Lucky-pre); after  
pancreatectomy but prior to transplantations (Lucky-  
20 post); and at intervals following transplantation (143  
days, 730 days and 930 days, respectively).

Figure 2 shows the C-peptide responses to an  
oral sustacal tolerance test at the same time intervals  
as depicted in Figure 1.

25 Figure 3 shows the glucose responses to oral  
sustacal tolerance tests in the monkey "Oscar".

Figure 4 shows the C-peptide responses in the  
same animal and at the same intervals depicted for  
Figure 3.

30 Figure 5a and 5b show the effect of  
intratesticular islet allografts on serum glucose levels  
and the insulin responses to oral glucose in



1 spontaneously diabetic BB/Wor dp rats. Figure 5a shows  
the plasma glucose (mg/dl) concentrations in response to  
the oral glucose administration of 2 g/kg of a 50%  
glucose solution in three groups of rats: untreated  
5 control Sprague Dawley, transplanted diabetic BB/Wor dp,  
and insulin treated diabetic BB/Wor dp rats. Figure 5b  
shows the serum insulin levels in response to the same  
dose of oral glucose in untreated control Sprague  
Dawley, and in transplanted BB/Wor dp rats.

10 Figures 6a and 6b show the effect of  
intratesticular islet allografts on plasma glucagon  
secretory responses to oral glucose and a combination of  
glucose plus glipizide in spontaneously diabetic BB/Wor  
dp rats. Figure 6a shows the plasma glucagon responses  
15 to the oral administration of 2 g/kg of a 50% glucose  
solution in three groups of rats: untreated control  
Sprague Dawley, transplanted diabetic BB/Wor dp, and  
insulin treated diabetic BB/Wor dp rats. Figure 6b  
shows the plasma glucagon responses to the oral  
20 administration of 7 mg/kg of glipizide and 2 g/kg of a  
50% glucose solution, administered 30 minutes later, in  
three groups of rats: untreated control Sprague Dawley,  
transplanted diabetic BB/Wor dp, and insulin treated  
diabetic BB/Wor dp rats. Data points are mean  $\pm$  SE of  
25 eight animals in each group.

Figure 7 shows a light micrograph of the  
pancreatic islets of Langerhans and the isolated rat  
Sertoli cells transplanted into the renal subcapsular  
space of a diabetic rat.

30 Figure 8 shows an electron micrograph of an  
individual cell within the transplanted islet.

Figure 9 shows an electron micrograph of the  
fine structure of the extra-islet cells labeled "S" in  
Figure 7.

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1           Figure 10 shows the effect of transplantation  
of piglet islets and Sertoli cells underneath the renal  
capsule on the mean daily urine output of seven grafted  
5       female rat recipients. Each bar represents the mean  
daily urine output over a ten-day period following  
transplantation.

          Figure 11 shows the effect of the  
transplantation of piglet islets and Sertoli cells  
underneath the skin on the mean daily urine volumes of  
10       three rats over a 50-day period.

          Figure 12 shows the light photomicrograph of  
pig islets of Langerhans and rat Sertoli cells  
transplanted into the renal subcapsular space of a  
diabetic rat. IL shows the presence of islands of beta  
15       cells (IL) surrounded by an infiltration of small  
lymphocytes underneath the renal capsule (K); B (upper  
left) shows at higher magnification that the islands  
(IL) consist of beta cells and B (lower right) shows  
that beta cells contain characteristic insulin granules.

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#### DETAILED DESCRIPTION OF THE INVENTION

          The present invention is directed to a method  
of treating a disease that results from a deficiency of  
a biological factor in mammals which comprises  
25       administering to a mammal Sertoli cells and cells that  
produce the biological factor. As defined by the  
present invention, a biological factor is a protein or  
nonprotein compound that is necessary for cellular  
metabolism and homeostasis. In a preferred embodiment,  
30       the biological factor is a hormone. Hormone producing  
cells which can be administered using the method  
described in the present invention include, for example,

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1     pancreatic islet of Langerhans, pituitary, liver,  
parathyroid and thyroid cells.

5             In accordance with the present invention, the  
Sertoli cells and the cells that produce the biological  
factor can be from the same species as the mammal to be  
treated or from a different species. Further, the  
Sertoli cells and the cells that produce the biological  
factor need not be derived from the same species. It  
has been demonstrated in accordance with the present  
10    invention that Sertoli cells from pigs in conjunction  
with islet of Langerhans from pigs can be used in the  
treatment of diabetes mellitus in rats. In a preferred  
embodiment the Sertoli cells are bovine, porcine or  
human.

15            Sertoli cells, which are the predominant cells  
of male testes, used in the method described by the  
present invention can be separated from other testicular  
cells such as Leydig cells, peritubular cells and germ  
cells, using conventional techniques. For example, the  
20    testes of a male mammal, such as a boar or ram, are  
first collected by castration. The testes are then  
chopped into several pieces and subsequently washed by  
centrifugation.

25            Testicular Leydig cells can be removed from  
the tissue suspension using digestion agents such as  
trypsin and DNase. The remaining cell suspension is  
then washed by centrifugation several times. The pellet  
is resuspended in collagenase, incubated and washed by  
centrifugation to eliminate peritubular cells within the  
30    testes. Testicular germ cells can be removed by  
incubating the pellet with hyaluronidase and DNase.  
After several washings by centrifugation, the Sertoli

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1 cells can be collected to transplant using the method of  
the present invention.

5 In accordance with the present invention, a  
biological factor is a protein or nonprotein compound  
that is absent, deficient or altered in a disease state.  
Cells that produce a biological factor can be isolated,  
for example, by first surgically removing the tissue  
that produces the factor from a mammal. This tissue is  
subsequently chopped and digested using conventional  
10 techniques. For example, the tissue can be digested  
using a collagenase digestion. The particular factor  
producing cells can subsequently be collected from the  
digestion mixture using a separation gradient such as a  
Ficoll gradient. The factor producing cells are then  
15 grown in tissue culture in serum using conventional  
techniques. The factor producing cells may be co-  
cultured with Sertoli cells in tissue culture. Cells  
grown in tissue culture can be transplanted into a  
mammal in conjunction with the Sertoli cells using the  
20 method of the present invention. In accordance with the  
present invention, factor producing cells may be stored  
using a variety of conventional techniques, such as  
cryopreserving the cells prior to growth in tissue  
culture for subsequent transplantation. It has been  
25 observed in accordance with the present invention, that  
Sertoli cells co-cultured with factor producing cells  
such as islet cells enhance the proliferation and  
recovery rate of the factor producing cells in tissue  
culture and in particular, enhance the recovery rate and  
30 proliferation of factor producing cells that have been  
previously stored using techniques such as  
cryopreservation.

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1           In a preferred embodiment the factor is a  
hormone, and the hormone producing cells are isolated  
from a tissue source as described above. For example,  
insulin-producing cells are isolated from the pancreas.  
5   In another preferred embodiment, the factor producing  
cells are provided by transforming suitable host cells  
with a nucleic acid capable of expressing the factor of  
interest. Transformed cells are provided by methods  
known to one of ordinary skill in the art, and can be  
10 found in a myriad of textbooks and laboratory mammals,  
including Sambrook et al. (1989) Molecular Cloning: A  
Laboratory Mammal, Cold Spring Harbor Laboratories, Cold  
Spring, New York. If necessary, the nucleic acid  
encoding the factor of interest can be adapted by  
15 methods known to one of ordinary skill in the art to  
effect secretion of the factor from the transformed  
cell. The utilization of Sertoli cells in conjunction  
with the factor producing cells in accordance with the  
method of the present invention allows the production of  
20 an immunologically privileged site in the treated  
mammal.

          The administration of factor producing cells  
and Sertoli cells into a mammal is accomplished by  
conventional techniques. In a preferred embodiment,  
25 administration is by transplantation and the factor  
producing cells are injected into the mammal  
concurrently with or immediately after the injection of  
the Sertoli cells into the same site. In accordance  
with the present invention, an exogenous biological  
30 factor may be administered following the transplantation  
of factor producing cells and Sertoli cells until the  
transplanted cells produce a therapeutically effective

1 amount of the biological factor. For the treatment of  
diabetes, for example, insulin may be administered  
following the transplantation of pancreatic islet cells  
and Sertoli cells until the transplanted islet cells  
5 produce a therapeutically effective amount of insulin.

The Sertoli cells and factor producing cells  
of the present invention can be transplanted using any  
technique capable of introducing the cells into the  
mammal such as parenteral administration or subcutaneous  
10 administration following surgical exposure to a desired  
site. Prior to transplantation, the recipient mammal is  
anesthetized using local or general anesthesia according  
to conventional technique. In a preferred embodiment  
the mammal to be treated is human. In another  
15 embodiment the present method of treating disease  
further comprises administering an immunosuppressive  
agent such as, for example, cyclosporine, tacrolimus,  
despergualin and monoclonal antibodies to, e.g., T  
cells. In a preferred embodiment the immunosuppressive  
20 agent is cyclosporine. In another preferred embodiment  
cyclosporine is administered at a dosage of from 0.5 mg  
to 200 mg/kg body weight. In a most preferred  
embodiment cyclosporine is administered at a dosage of  
from 5 mg to 40 mg/kg body weight.

25 It has been discovered in accordance with the  
present invention that administration of Sertoli cells  
and factor producing cells results in the creation of an  
immunologically privileged site in the treated mammal.  
An immunologically privileged site as defined by the  
30 present invention is a site in the mammal where the  
immune response produced in response to the transplanted  
cells is suppressed due to immuno-suppressive agents

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1 produced by Sertoli cells. Immunologically privileged  
sites are characterized by an available blood supply to  
provide nourishment for the transplanted cells and a  
dense tissue to keep the transplanted cells within close  
5 proximity of each other. Examples of immunologically  
privileged sites as defined by the present invention  
include the renal subcapsular space, subcutaneous facie,  
the brain and the hepatic portal vein.

10 In accordance with the present invention it  
has been shown that Sertoli cells increase the rate at  
which the transplanted factor producing cells  
vascularize in the transplanted site. It is therefore  
indicated that the Sertoli cells (i.e. the relevant  
agents produced by the Sertoli cells) promote the  
15 increased vascularization rate of the transplanted  
cells, for example, islet cells).

20 In a preferred embodiment, the present  
invention describes a method of treating diabetes  
mellitus by transplanting islet of Langerhans in  
conjunction with Sertoli cells to create an  
immunologically privileged site. Allografts as used in  
the present invention describes the transfer of tissues  
or cells between two genetically dissimilar mammals of  
the same species. The term xenografts in the present  
25 invention describes the transfer of tissues or cells  
between two mammals of different species.

30 The transplanted islet of Langerhans cells and  
Sertoli cells used in the method described by the  
present invention can be prepared using any number of  
conventional techniques. For example, islet of  
Langerhans cells can be prepared from the pancreas of  
several mammals of the same species. The pancreases are  
pooled together, chopped up and digested using

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1 collagenase. The islet of Langerhans cells can be  
further isolated using conventional gradients. Once  
isolated, the islet cells can be grown in culture and  
then transplanted in conjunction with Sertoli cells to  
5 create an immunoprivileged site.

Sertoli cells used in the method described by  
the present invention can be isolated from mammalian  
male testes. To collect the islet cells, the testes are  
first chopped into several pieces and then washed by  
10 centrifugation. Leydig cells, present in the crude  
mixture, can be removed from the tissue suspension using  
digestion agents such as trypsin and DNase. The  
remaining cell suspension is then washed by  
centrifugation several times. Following, the pellet may  
15 be resuspended in collagenase, incubated and washed by  
centrifugation to eliminate peritubular cells within the  
testes. Testicular germ cells can be removed by  
incubating the pellet with hyaluronidase and DNase.  
After several washings by centrifugation, the Sertoli  
20 cells for transplantation can be collected.

The Sertoli cells can be transplanted to  
create an immunoprivileged site within a mammal using a  
variety of techniques. For example, after the mammal is  
anesthetized, the Sertoli cells can be injected into a  
25 tissue mass, thereby creating an immunoprivileged site.

Sertoli cells are administered in an amount  
effective to provide an immunologically privileged site.  
Such an effective amount is defined as that which  
prevents immune rejection of the subsequently or co-  
30 administered cells that produce the biological factor.  
Immune rejection can be determined for example



1 histologically, or by functional assessment of the  
factor produced by the cells.

In a preferred embodiment Sertoli cells are  
administered in amounts ranging from  $10^1$  to  $10^{10}$  cells.

5 In a more preferred embodiment,  $10^5$  to  $10^{10}$  cells are  
administered.

10 The cells producing the biological factor are  
administered in a therapeutically effective amount. The  
ordinary skilled artisan can determine the appropriate  
amount of cells producing the biological factor by  
methods known in the art. The amount of cells is  
dependent upon the amount of factor being produced by  
the cells and the known therapeutically effective amount  
of the factor necessary to treat the disease. For  
15 example, 1 to 1000 islet cells per gram body weight can  
be administered to treat diabetes using allografts, 20  
to 1000 islets per gram body weight are administered  
using xenografts. In another preferred embodiment, 5 to  
100 islet cells per gram body weight are administered to  
20 treat diabetes. In a most preferred embodiment, 5 to 20  
islet cells per gram body weight are administered, using  
allografts and 100-1000 islet cells per gram body weight  
are administered for xenografts.

25 In another embodiment the present method of  
treating diabetes further comprises administering an  
immunosuppressive agent such as, for example,  
cyclosporine, tacrolimus, desfergualin and monoclonal  
antibodies to, e.g., T cells. In a preferred embodiment  
the immunosuppressive agent is cyclosporine. In another  
30 preferred embodiment cyclosporine is administered at a  
dosage of from 0.5 mg to 200 mg/kg body weight. In a

1 most preferred embodiment cyclosporine is administered  
at a dosage of from 5 mg to 40 mg/kg body weight.

5 More generally, the immunosuppressive agent  
can be administered for a time sufficient to permit the  
transplanted islets to be functional. This period  
extends from the point prior to or immediately following  
the transplantation of the islets to the point at which  
the cells are capable of producing therapeutically  
effective amounts of insulin. In a preferred  
10 embodiment, the sufficient period of time to administer  
an immunosuppressive agent is about 40 to about 100 days  
following transplantation of the islets. In a more  
preferred embodiment, the sufficient period of time is  
about 50-60 days.

15 A preferred embodiment of this invention is  
directed to a method of treating Type I and Type II  
diabetes mellitus by transplanting islet of Langerhans  
in conjunction with Sertoli cells into the renal  
subcapsular space.

20 Unlike the therapies for diabetes described in  
the prior art, the method of treating diabetes described  
by the present invention prevents the complications of  
the disease process and does not result in the adverse  
side effects associated with conventional diabetes  
25 therapy. Furthermore, the method of transplanting islet  
cells described by the present invention provides the  
necessary factors for angiogenesis of the islet  
transplants.

30 A method of creating an immunologically  
privileged site in a mammal is further described by the  
present invention. An immunologically privileged site  
is created by transplanting isolated Sertoli cells into

1 a mammal in an amount effective to create an  
immunologically privileged site. In a preferred  
embodiment,  $10^1$  to  $10^{10}$  cells are administered. In a  
5 administered. In a preferred embodiment the Sertoli  
cells are transplanted into the renal subcapsular space  
or subcutaneous facie by injection. In a preferred  
embodiment the mammal is a human and the Sertoli cells  
are human or porcine.

10 Further contemplated in accordance with the  
present invention is a method of enhancing the recovery  
and proliferation of ex vivo cells comprising co-  
culturing said cells with Sertoli cells for a time and  
under conditions sufficient to achieve said enhanced  
15 recovery and proliferation.

Another aspect of the present invention  
provides a pharmaceutical composition comprising Sertoli  
cells and cells producing a biological factor and a  
pharmaceutically acceptable carrier. In a preferred  
20 embodiment the composition comprises Sertoli cells and  
islet of Langerhans cells and a pharmaceutically  
acceptable carrier. A further preferred embodiment of  
the present invention comprises using porcine, bovine or  
human Sertoli cells and porcine, bovine or human islet  
25 of Langerhans cells. As used herein, a pharmaceutically  
acceptable carrier includes any and all solvents,  
dispersion media, coatings, antibacterial and antifungal  
agents, isotonic agents and the like. The use of such  
media and agents is well-known in the art. The present  
30 invention further contemplates a pharmaceutical  
composition comprising Sertoli cells and a  
pharmaceutically acceptable carrier.

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1           The present invention is also directed to a  
kit for treatment of a disease. In one embodiment, the  
kit is compartmentalized to receive a first container  
5       adapted to contain Sertoli cells in an amount effective  
to create an immunologically privileged site in a  
mammal, and a second container adapted to contain a  
therapeutically effective amount of cells that produce a  
biological factor that is absent or defective in the  
10       disease to be treated. In a preferred embodiment, the  
Sertoli cells are bovine, porcine or human and are  
provided in an amount of from  $10^1$  to  $10^{10}$  cells. In a  
more preferred embodiment, Sertoli cells are provided in  
an amount of from  $10^5$  to  $10^{10}$  cells. In another  
15       preferred embodiment the cells that produce a biological  
factor are cells that have been transformed with DNA  
encoding the factor. In yet another preferred  
embodiment the cells that produce the factor are  
pancreatic islet cells. The islet cells are provided in  
20       a preferred amount of 5 to 200 cells per gram of body  
weight, and in a more preferred amount of 5 to 100 cells  
per gram of body weight.

          The present invention further provides an  
article of manufacture comprising a packaging material  
and Sertoli cells contained within said packaging  
25       material, wherein said Sertoli cells are effective for  
creating an immunologically privileged site in a mammal,  
and wherein said packaging material contains a label  
that indicates that said Sertoli cells can be used for  
creating an immunologically privileged site in a mammal.  
30       The packaging material used to contain the Sertoli cells  
can comprise glass, plastic, metal or any other suitably  
inert material.

1           In order to further illustrate the present  
invention, the experiments described in the following  
examples were carried out. It should be understood that  
the invention is not limited to the specific examples or  
5 the details described therein. The results obtained  
from the experiments described in the examples are shown  
in the accompanying figures and tables.

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EXAMPLE 1

Six male Rhesus monkeys were transplanted with islet allografts in their testes to examine the survival of these transplants. The recipients were made diabetic by means of a near total pancreatectomy, followed two weeks later by an intravenous injection of 35 mg streptozotocin/kg body weight. This procedure resulted in the induction of severe diabetes melitis. Plasma glucose levels were in excess of 400 mg/dl and the animals were ketotic. Malabsorption was prevented by the oral administration of VIOKASE®, one tablet given twice daily before each meal.

Islets were isolated from female Rhesus monkeys. First, the pancreases of five animals were removed, pooled and chopped finely into smaller fragments. After collagenase digestion in a water bath at 37°C, the islets were separated from exocrine tissues and other cellular debris on at least two Ficoll gradients, prepared in tandem. The islets were washed three times by centrifugation in ice-cold Hanks's buffer and then handpicked and transferred in groups of 150 to biologic grade Petri dishes. Each dish contained 6 mL of culture medium CMRL-1066 supplemented with 5% fetal calf serum, glucose at a concentration of 250 mg/dL, penicillin (100 U/mL), and streptomycin (100 µg/mL). Incubation of islets were carried out at 35°C in 5% CO<sub>2</sub> and air for 4 to 6 days. The islets were transferred to fresh medium at 48 hour intervals.

Viability and counting of the islets were facilitated by means of the uptake of the dye dithizone. Each monkey received an average of about 10<sup>4</sup> islets/kg

1 body weight injected into both testes. In the first  
three animals the testes were elevated into the  
abdominal cavity, whereas in the last three recipients  
the grafted organs were anchored into the inguinal  
5 canal. Cyclosporine (CsA) was administered, in varying  
doses to the first three grafted animals over a 30 day  
period, whereas the last three hosts were given 7  
injections of CsA (20 mg/kg) on days -4 to +3. Oral  
sustacal tolerance tests were done on day 30, and then  
10 at intervals in the normoglycemic animals, as follows.

The monkeys were housed individually in cages  
and given standard monkey chow and fruit twice daily.  
In addition, a pancreatic enzyme was mixed with the food  
since the monkeys had been pancreatectomized to make  
15 them diabetic before transplantation.

The night before the test, the animals were  
fasted for 12 hours. At 8 a.m. the next morning they  
were then anesthetized and prepared for the test meal.  
Sustacal was used as the test agent. Sustacal consists  
20 of a physiologic mixture of carbohydrates, proteins and  
fat which closely mimics a standard meal and which is a  
powerful stimulus for the release of insulin.

Sustacal was injected directly into the  
stomach of the sleeping animal through a nasogastric  
25 tube. Blood samples were then obtained at times 0, 15,  
30, 60, 90, 120 and 180 minutes. The samples were  
centrifuged and the serum stored at -20°C until  
measurements for insulin or C-peptide could be carried  
out. C-peptide is a very sensitive marker for beta cell  
30 function. The results are shown in Figures 1-4.

Figure 1 shows the glucose responses to oral  
sustacal tolerance tests done on the monkey "Lucky" at

1 intervals before pancreatectomy (Lucky-pre); after  
pancreatectomy but prior to transplantation (Lucky-  
post); and at intervals following transplantation (143  
days, 730 days and 930 days, respectively).

5 It can be readily appreciated that the animal  
became severely diabetic after the removal of his  
pancreas (Lucky-post). Following transplantation the  
glucose responses were restored to normal levels at all  
10 of the time intervals measured (143, 730 and 930 days  
following transplantation). Lucky showed no evidence of  
graft failure. With graft failure glucose levels would  
become elevated would approach those which were found  
following his pancreatectomy.

15 Figure 2 shows the C-peptide responses to an  
oral sustacal tolerance test at the same time intervals  
as depicted in Figure 1. Following his pancreatectomy  
the C-peptide responses became blunted indicating a  
severe diabetes. But following transplantation the  
levels were not only restored to normal but appeared to  
20 show a "hyperresponsive" pattern of C-peptide release  
and levels done on day 730 exceed the normal levels at  
all points measured. The elevated levels might be due  
to the fact that insulin released from the testis enters  
the systemic circulation. By contrast, insulin released  
25 from the pancreas enters the portal vein and travels  
immediately to the liver where about 60% is broken down  
during the first passage. Insulin released into the  
systemic circulation reaches the liver much later, thus  
the elevated levels. As was evident with an  
30 investigation of the glucose concentrations, the C-  
peptide responses showed no evidence of failure 30  
months following transplantation.



1           Figure 3 shows the glucose responses to oral  
sustacal tolerance tests in the monkey "Oscar".  
Following the removal of his pancreas he became severely  
diabetic with elevated glucose levels. Following  
5   transplantation of islets the glucose responses became  
similar to those determined before his pancreas was  
removed. The glucose levels remain within normal levels  
32 months following transplantation.

10           Figure 4 shows the C-peptide responses in the  
same animal and at the same intervals depicted for  
Figure 3. The animal became very diabetic following the  
removal of his pancreas and shows blunted C-peptide  
responses as a result. Following transplantation and  
for the next 730 days the C-peptide responses were  
15   greater compared with the normals. On day 930 following  
transplantation the C-peptide responses have become  
somewhat less compared with the normals. Despite  
somewhat lower C-peptide levels the animal remains  
normoglycemic.

20           This example demonstrates that primates can be  
successfully transplanted with intratesticular islet  
allografts without the need for sustained  
immunosuppression, and that functional integrity of  
intratesticular islet allografts is maintained for  
25   periods exceeding two years with no evidence of graft  
failure.

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EXAMPLE 2

This study examined insulin and glucagon secretory patterns in spontaneously diabetic bb/Wor dp rats transplanted with abdominal, intratesticular, islet grafts. Diabetic, BB/Wor dp, rats received intratesticular islet grafts from MHC-compatible BB/Wor dr rats and no immunosuppression. After a period of 74±15 days, of normoglycemia, three different groups (controls; BB/Wor dp, transplanted; and BB/Wor dp, insulin treated) were given the following challenges; (1) an oral glucose tolerance test (OGTT), (2) a single oral dose of glipizide, followed by an OGTT, and (3) arginine, by intravenous infusion. The results of this study are shown in Tables 1 and 2 and Figures 5 and 6.

TABLE 1

Metabolic Parameters and Immunoreactive Serum Insulin and Glucagon Levels in Control, and in Transplanted and Insulin treated, BB/Wor dp Rats.

	Controls	BB/Wor dp Grafted*	Insulin treated
Plasma Glucose (mg/dl):			
Prior to therapy	112 ± 5	502 ± 8+	510 ± 13+
After 2.5 months	97 ± 4	110 ± 3	350 ± 40 #
Duration p.t. OGTT (days)	75 ± 6	70 ± 11	78 ± 19
Weight gain (g)	120 ± 6	105 ± 17	48 ± 14 \$
Fasting Plasma Insulin (uU/ml)	21.9 ± 3	20.4 ± 2	ND
Fasting Plasma Glucagon (pg/ml)	37.8 ± 5.7	43.4 ± 4.6	47.4 ± 4.9

\* Duration of normoglycemia after grafting (days) = 279 ± 25; + P < 0.0001 vs. control

# P < 0.0001 vs. grafted; \$ P < 0.02 vs. grafted

**TABLE 2**

Pancreatic and Testicular Insulin and Glucagon Content in Control, and in Transplanted and Insulin Treated, BB/Wor dp, Rats.

	Controls	BB/Wor dp Grafted	Insulin treated
Pancreas (mg)	1573 ± 171	757 ± 122	920 ± 32
Insulin (ug/g)	66 ± 5.03	0.58 ± 0.18	0.76 ± 0.12
Glucagon (ng/mg)	4.1 ± 0.35*	4.9 ± 0.33**	6.9 ± 0.80
Testes fractions: (mg)	493 ± 49.6	582 ± 59.2	430 ± 28.0
Insulin (ug/g)	0.0	59.70 ± 0.49	0.0
Glucagon (ng/mg)	0.0	1.4 ± 0.37	0.0

\* P < 0.03 and \*\* P < 0.08, vs. diabetic, respectively

Figure 5 shows the effect of intratesticular islet allografts on serum glucose and insulin responses to oral glucose in spontaneously diabetic BB/Wor dp rats. Figure 6 shows the effect of intratesticular islet allografts on plasma glucagon secretory responses to oral glucose and a combination of glucose plus glipizide in spontaneously diabetic BB/Wor dp rats. This experiment demonstrates that grafted testes in spontaneously diabetic BB/Wor dp rats contain both alpha and beta cells, and that the alpha and beta cells have the capacity to respond to specific secretagogues independently.

EXAMPLE 3

This study investigated the effect of Sertoli cell-enriched fraction (SEF) on islet allograft survival in the renal subcapsular space of diabetic rats.

The animals used in this study were PVG rats, weighing between 150-200 g. Diabetes was induced by means of a single intravenous injection of 65 mg/dL of streptozotocin. Only rats with plasma glucose levels in excess of 400 mg/dL were transplanted. Sprague Dawley (S-D) outbred rats were used as islet donors. Either PVG or S-D male rats between 16 and 18 days old were used as Sertoli cell donors.

**Islet Preparation**

Islets were prepared according to modification of the method of London et al. (1990) Transplantation, 49: 1109-1113. The islets were purified on Ficoll gradients, and the isolated cells were then incubated for 4 days at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and air prior to use. No special efforts were made to deplete the islets of contaminating passenger leukocytes.

**Sertoli Cell-enriched Fraction Preparation**

Highly purified preparations of Sertoli cells were isolated from the testes of young males according to the method of Cheng et al. J. Biol. Chem., 26:12768-12779. The testes were removed, chopped into several pieces, and placed in a 50 mL conical tube containing 50 mL of Ham's F12/DMEM media. The pieces were washed once by centrifugation at 800 x g for 2 min. The supernatant

1 was aspirated, and the tissue resuspended in 40 mL of  
media containing 40 mg trypsin and 0.8 mg DNase in a  
sterile 250 mL Erlenmeyer flask. The flask was placed  
5 in 37°C oscillating incubator at 60-90 osc/min for 30  
min. This step removed Leydig cells. The tubules were  
then transferred to a 50 mL conical tube, and  
centrifuged at 800 x g for 2 min. The supernatant  
fraction was aspirated, and the pellet resuspended in 40  
10 mL of 1 M glycine, 2 mM EDTA containing 0.01% soy bean  
trypsin inhibitor and 0.8 mg DNase, and incubated at  
room temperature for 10 min. This step lysed any  
residual Leydig cells. The cells were washed by  
centrifugation for 2 min, and the step repeated twice,  
or until the media was no longer cloudy. The pellet was  
15 resuspended by gentle homogenization with a glass  
Pasteur pipet in 40 mL of media containing 20 mg  
collagenase in an Erlenmeyer flask, and incubated at  
37°C for 5 min with 60-90 osc/min. The cell suspension  
was centrifuged at 800 x g for two min, and the pellet  
20 resuspended by gentle homogenization with a Pasteur  
pipet in 40 mL media containing 40 mg collagenase and  
0.2 mg DNase, and incubated in an Erlenmeyer flask at  
37°C for 30 min with 60-90 osc/min. The cells were then  
washed by centrifugation for 2 min, and the process  
25 repeated at least three times to eliminate peritubular  
cells. The cells were resuspended by gentle  
homogenization with a Pasteur pipet in 40 mL media  
containing 40 mg hyaluronidase and 0.2 mg of DNase, and  
incubated at 37°C for 30 min with 60-90 osc/min. The  
30 cells were pelleted by soft centrifugation for 2 min,  
and washed at least five times to eliminate germ cells.  
The resultant SEF was resuspended in 0.25 mL of media,

1 and immediately transplanted into the recipient rat.  
Each grafted rat received the equivalent of the total  
amount of Sertoli cells contained in a single testis.

#### 5 Transplantation of Rats

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The diabetic rat was anesthetized with  
methoxyflurane USP in a sterile hood and the left flank  
opened to expose the kidney. The Sertoli-enriched  
fraction containing approximately 5 million Sertoli  
10 cells was injected first underneath the renal capsule.  
The cells could be seen as a milkish bubble underneath  
the capsule. Immediately afterwards, a total of 10  
islets/g of body weight was injected to the same milkish  
bubble. The needle was retracted slowly to prevent  
15 leakage of the grafted cells. Cyclosporine (CsA) was  
administered subcutaneously in varying doses over a 20-  
day period to groups two and four. Because the grafted  
rats responded similarly whether the drug was  
administered over a 20-day, or over a 3-day period, all  
20 of the subsequent groups, including the female rats,  
were treated with only three injections of 25 mg/kg CsA,  
given on days 0, +1, and +2, relative to the graft. The  
rats received no other therapy.

A total of 36 male and 21 female PVG rats were  
25 divided into six different treatment groups: Group 1,  
the control group, consisted of 6 male rats grafted with  
only islets from S-D donor rats. They received neither  
SEF nor CsA. Group 2 consisted of 10 rats grafted with a  
combination of islets from S-D rats and CsA  
30 posttransplantation, but no SEF. Group 3 consisted of a  
total of 10 rats grafted with a combination of islets  
from S-D and SEF from PVG donor rats, but no CsA

1 postransplantation. Group 4 consisted of 10 rats  
grafted with a combination of islets from S-D donors,  
SEF from PVG donors, and CsA postransplantation. Group  
5 consisted of 11 female rats grafted with the same  
5 combination of cells as depicted for Group four. Group  
6 consisted of 10 female rats grafted with a combination  
of islets and SEF, both cell types from S-D donors, and  
CsA postransplantation.

10 **Postransplantation Evaluation of Rats**

The grafted rats were transferred to metabolic  
cages, and plasma glucose levels were obtained at weekly  
intervals. Urine volumes and urine glucose contents  
were obtained at daily intervals. A rat was considered  
15 cured of the diabetic process if the following criteria  
were met: A random plasma glucose level  $\leq 150$  mg/DL;  
glycosuria; and immediate reversal to hyperglycemia  
following surgical removal of the grafted kidney.

To determine if any of the rats had become  
20 unresponsive to their grafts, normoglycemic rats were  
challenged with a secondary islet allograft consisting  
of at least 500, freshly prepared, Sprague Dawley islets  
which were injected into the contralateral renal  
subcapsular space. No immunosuppression was given  
25 following the challenge.

To examine the impact of the transplantation  
of SEF on fertility of the female rats, normoglycemic  
animals of longer than 30 days were mated with PVG  
males. Metabolic parameters, as outlined above, were  
30 closely monitored, as was the course of their  
pregnancies.

# 1     **Structural Analysis of Grafted Tissue**

5           A total of five successfully grafted rats were  
nephrectomized at intervals following transplantation.  
10     Wedge sections of renal tissue, obtained from sites at  
which islets and SEF had been injected, were prepared  
for examination by light and electron microscopy, as  
previously described by Cameron et al. (1990)  
Transplantation, 50:649-653. Briefly, the tissue wedges  
15     were immersion-fixed with 5% glutaraldehyde in 0.1 M s-  
Collidine buffer for 1 h, washed in buffer, and post-  
fixed for 1 h with 1% osmium tetroxide in 0.1 M buffer.  
Small tissue blocks were cut from the wedges, and  
20     dehydrated through a graded series of ethyl alcohols,  
transferred to propylene oxide, and embedded in Epon  
812/Araldite plastic resin. Thick (0.5µm) and thin (900  
mg) sections were stained routinely with toluidine blue  
and uranyl acetate/lead citrate, respectively, for  
structural analysis by light and electron microscopy.  
The results are shown in Table 3 and Figs. 7-9.

**TABLE 3**

Effect of Sertoli cells on islet allograft survival in  
the non-immunologically privileged renal, subcapsular  
site

Group(n)	Gender	Sertoli cell (donor origin)	CsA	Duration of normoglycemia (days) Individual Responses
1 (6)	Male	--	-	0,0,0,0,0,0
2 (10)	Male	--	+	0,0,0,0,0,0,130 > 441, > 445
3 (10)	Male	+ (FVG)	-	0,0,0,0,9,10,12,13,13,14
4 (10)	Male	+ (FVG)	+	19,76,58*,84*,167*,127†,139†,>418†,>422†, >425†
5 (11)	Female	+ (FVG)	+	7,11,14,28,>287†,>305†,>306†,>308†,>441†,>447†, >457†
6 (10)	Female	+ (S-D)	+	8,10,96*,128*,>168,>172,>184,>193,>193,>196

\* nephrectomized, †challenged with a secondary islet allograft.



1           Group 1: None of the six rats grafted with  
islets alone, without either SEF or CsA, became  
normoglycemic.

5           Group 2: Three of 10 rats grafted with islets  
and treated with CsA became normoglycemic for more than  
100 days. The 3 normoglycemic rats were challenged with  
a secondary graft on days 116, 192 and 197,  
respectively. One rat reverted to hyperglycemia on day  
130, while 2 remained normoglycemic.

10          Group 3: Initially 6 of the 10 rats grafted  
with islets and SEF, but no CsA, became normoglycemic,  
but all of them reverted to hyperglycemia by day 14.

15          Group 4: All 10 of rats grafted with a  
combination of SEF and islets, and also given CsA became  
normoglycemic. Two reverted spontaneously to diabetes  
on days 19 and 76, respectively. Three were  
nephrectomized on days 58, 84 and 167 following  
transplantation. All 3 of these rats became  
hyperglycemic within the next 24 h. The remaining 5  
20 rats were challenged with a secondary islet allograft on  
days 119, 129, 280 ,342 and 400, respectively. Of  
these, the first 2 reverted to diabetes on day 127 and  
139, respectively, while the latter 3 remained  
normoglycemic.

25          Group 5: All 11 of the female rats grafted  
with a combination of islets and SEF, and then given  
CsA, became normoglycemic. Of these, 4 reverted  
spontaneously to hyperglycemia by day 28. Of the 7  
normoglycemic rats who were mated with male PVG rats, 6  
30 became pregnant, and of these, 8 had litters varying  
between 1 and 10 pups. They were able to nurse the pups  
successfully. A total of 7 of the long-term surviving

1 females were challenged with secondary islet allografts  
at least 200 days following transplantation. None of  
them reverted to hyperglycemia.

5 Group 6: Of the 10 rats grafted with islets  
and SEF from the same donor strain of rat, all 10 became  
normoglycemic. Two reverted to hyperglycemia by day 10.  
A nephrectomy to remove the graft was done on 2 of the  
long-term surviving rats on days 96 and 201,  
respectively. Both reverted to hyperglycemic  
10 immediately within the next 24 h.

### **Tissue Morphology**

Renal tissue obtained from the long-term  
grafted kidney appeared structurally normal by light  
15 microscopy (Figure 7). Transplanted islets in this  
organ were immediately subjacent to the kidney capsule,  
and also appeared structurally normal. They displayed  
tissue and cellular architecture identical to islets in  
situ (Figure 7). Individual islet cells were  
20 partitioned into cell clusters by thin connective septa  
containing small vessels and capillaries (Figure 7). It  
appeared that most of the islet cells contained  
secretion granules. When resolved by electron  
microscopy, islet cells were identified as the  $\beta$ -cell  
25 type by the inclusion of ultrastructurally distinctive,  
and unique insulin-containing secretion granules (Figure  
8). All  $\beta$ -cell clusters observed were in close  
proximity to intra-islet capillaries (Figure 8).

30 There was a high density of cells between, and  
directly adjacent to, the transplanted islets and renal  
parenchyma. By light microscopy, they did not appear to  
be islet cells, kidney cells nor cells of blood origin

1 (Figure 7). When observed by electron microscopy, these  
cells were similar in ultrastructure to Sertoli cells in  
that their nucleic were irregular in profile, and  
5 contained deep nuclear clefts, distinctive nucleoli were  
often present, and mitochondrial structure was dense.  
Although these cells did not retain the typical polarity  
of Sertoli cells in vivo, they were, however, identical  
in appearance to Sertoli cells in vitro, when the cells  
are not plated on a basement membrane substrate. The  
10 cells were not associated with a basement membrane, and  
appeared randomly organized (Figure 9). Cells showing  
ultrastructural features of either germ or Leydig cells  
were not observed.

This example demonstrates that an  
15 immunologically privileged site for transplantation of  
isolated islet can be created in male and female  
diabetic recipients by transplantation of Sertoli cells  
without the need for sustained immunosuppression.

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EXAMPLE 4

This study determined the survival of discordant islet xenografts in various nonimmunologically privileged organ sites in experimental animals.

Islets were prepared from young piglets as follows: Male piglets not weighing more than 2.2 kg were used exclusively. The piglet was anesthetized and following exsanguination both pancreas and testes were harvested under sterile conditions. A collagenase solution consisting of 2 mg/ml of collagenase type XI (Sigma) was injected directly into the pancreas. The pancreas was incubated at 37°C for 17 minutes and the digested tissues washed three times by means of centrifugation and aliquots of 1 ml each transferred to Petri dishes. The islets were incubated at 32°C in tissue culture media 199 supplemented with 10% horse serum for six days.

On day seven the cultured islets were collected in batches of  $\pm 4,000$  and cryopreserved using a standard protocol. The cells were stored in liquid nitrogen at -96°C for periods varying between two and four weeks. The islets were removed from the liquid nitrogen and thawed using an established procedure. The thawed islets were transferred to Petri dishes and co-cultured with pig Sertoli cells for three days at 32°C in the same 199 culture media as described above. Earlier studies have shown an improved survival rate of thawed islets cultured in the presence of Sertoli cells.

On day three following thawing the islets were hand-picked and counted and a total amount of 12 islets/g of body weight transplanted into female

1 diabetic Sprague Dawley rats. A total of 5 million  
Sertoli cells procured from the piglet testes were  
grafted simultaneously into the same location. The  
organ sites to be tested for the grafting of islets  
5 include: a] the renal subcapsular space, b]  
subcutaneously, and c] the liver. Following  
transplantation, the rats were treated with cyclosporine  
as follows: 25 mg/kg for 7 days; 15 mg/kg for 5 days;  
10 mg/kg for 5 days; 5 mg/kg for an additional 13 days.  
10 On day 30 the drug was discontinued.

To demonstrate viability and functional  
integrity of isolated piglet islets the following  
studies were done: a) staining of Cells with dithizone,  
a stain which is highly specific for insulin; b)  
15 staining of cells with 0.4% trypan blue which indicates  
viability of the islets; and c) culturing of batches of  
5 islets in the presence of insulin secretagogues such  
as low and high glucose concentrations at specified  
intervals following culturing, cryopreservation and  
20 thawing. The results are shown in Table 4.

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**TABLE 4**

Insulin secretion (micro-units/ml) from incubated and from cryopreserved-thawed islets done on days 3, and 7, and 14, of culturing, respectively.

	Micro-units of	Insulin release	per 5 Islets
	Days following	isolation and	incubation
	3 Days	7 Days	14 Days
Incubated islets prior to cryopreservation:			
a) Low glucose (90 mg/dl)	15.3 ± 3.8	21.8 ± 1.1	17.29 ± 2.4
b) High glucose (300 mg/dl)	32.2 ± 5.4	37.14 ± 3.4	23.3 ± 1.8
Cryopreserved and thawed islets			
a) Low glucose (90 mg/dl)	14.52 ± 2.8	7.13 ± 1.3	5.38 ± 2.02
b) Low glucose + Sertoli cells	10.31 ± 2.8	9.17 ± 2.6	8.38 ± .41

**TABLE 5**

Yield of porcine islets following 1, 3, and 7 days of culture and the percentage of islets lost during 7 days of culture.

Pig. No.	BW (kg)	Panc W g	D1 islets /g panc	D3 islets /g panc	D7 islets /g panc	islet loss % D7/D1
1	1.6	1.79	36,536	31,659	27,212	26%
2	2.0	1.89	37,272	32,962	27,883	25%
3	2.3	2.46	29,268	26,046	20,884	29%
4	1.8	1.66	39,904	37,726	31,664	21%
5	1.8	1.76	37,846	34,578	30,046	21%
6	1.6	1.74	39,866	37,888	32,424	19%
7	1.4	1.61	42,126	39,456	33,872	20%
8	2.3	2.48	33,682	29,334	24,892	26%
9	2.1	2.28	43,478	41,226	37,394	14%
10	2.1	2.09	40,126	36,448	33,282	17%
11	2.1	2.12	31,248	27,170	26,415	15%
12	2.1	1.98	38,848	36,465	29,293	25%
13	2.2	2.06	39,146	37,446	31,709	19%
14	2.2	2.24	27,892	25,028	21,342	23%
15	2.7	2.69	44,610	38,364	31,524	29%
16	1.5	1.44	42,222	40,414	31,244	26%
Mean ± SE	2.0 ± 0.3	2.0 ± 0.4	37692 ± 1233	34513 ± 1307	29442 ± 1119	22.2 ± 1.2%

**TABLE 6**

Recovery of islets following freezing and thawing in presence and absence of Sertoli cells

	Islets alone			Islets + Sertoli cells		
	No. of islets	Pre-cryo	Post thawing Recovery (%)	Pre-cryo	Post thawing Recovery (%)	
D3F/D3T	250	152	61%	290	212	73%
	230	131	57%	260	228	88%
	440	278	63%	430	380	88%
	420	366	87%	410	324	79%
	450	290	64%	440	358	81%
		Means	66.4%			81.8%
D7F/D3T	260	136	52%	250	229	92%
	300	208	69%	300	202	67%
	280	177	63%	290	238	82%
	360	205	57%	350	300	86%
	320	218	68%	390	289	74%
	380	217	57%	320	270	84%
		Means	61.0%			80.8%

As shown in Table 5, the yield of islets per gram pancreas was  $37692 \pm 1233$ ,  $34513 \pm 1307$  and  $29,442 \pm 1119$ , after 1, 3 and 7 days of culture, respectively. Following cryopreservation and thawing and reculturing of the cells in the presence of Sertoli cells approximately 20% of the cells were damaged or lost as shown in Table 6. Thus  $\pm 24,000$  islets/gram of piglet pancreas were available for transplant purposes after cryopreservation and thawing.

The results showed that insulin secretion was blunted when glucose was used as insulin secretagogue prior to cryopreservation. The effect was more evident following cryopreservation and thawing. While the



1 presence of Sertoli cells had marked effects on number  
of islets that survived cryopreservation and thawing  
their presence had little effect on the ability of the  
islets to respond to a low glucose concentration as  
5 insulin releasing agent. However, as shown in Example 8  
the presence of Sertoli cells augmented the secretion of  
insulin in the presence of high glucose concentrations  
and glucose plus Forskolin.

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EXAMPLE 5

**Response of Diabetic Sprague  
Dawley Rats to the Transplantation of  
Islets from Piglet Donors (Discordant Xenografts)**

The rats were made diabetic by means of a single i.v. injection of 55 mg/kg of streptozotocin. They were grafted only if the blood sugar was equal to or more than 400 mg/dl. Following transplantation the rats were placed individually in metabolic cages and urine volume, urine glucose content, and body weights were measured at daily intervals. Blood glucose levels were done at weekly intervals. A rat is considered cured of diabetes if the blood glucose level is 160 mg/dl or less and/or the daily urine volume is 15 ml or less.

The results are illustrated in Figures 10 and 11.

Figure 10 shows the effect of transplantation of piglet islets and Sertoli cells underneath the renal capsule on the mean daily urine output of seven grafted female rat recipients. Each bar represents the mean daily urine output over a ten-day period following transplantation. The study has been conducted over an 80-day period, the bar on the furthest right thus showing the mean urine output per day from day 80 through 89, etc.

The figure shows that the mean daily urine volume for the first 60 days varied between 19.7 mls and 27 mls or within a diabetic range. It can be readily appreciated that urine volumes decreased to near-normal levels only from days 70 through day 89. The corresponding plasma glucose levels during the first and

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EXAMPLE 6

This study determined the effect of cultured Sertoli cells on the survival of discordant islet xenografts in diabetic rats with minimal early exogenous immunosuppression.

Preparation of Islets

Neonatal piglets of less than seven days of age were killed by anesthesia and islets were isolated according to a method of Kuo C.Y., Burghen G.A., Myvack A. and Herrod H.G. (1994) "Isolation of islets from neonatal pig pancreatic tissue", J. Tissue Culture Methods, 16:1-7. Briefly, the pancreas was distended by an injection of a collagenase solution, 2 mg/ml, collagenase type X1, in culture medium DMEM. After incubation at 39°C for 17 min, the digested fragments were washed by centrifugation and the digested tissue was then incubated for one week in medium 199 supplemented with 10% horse serum and 1% antibiotics at 32°C. The islets were then cryopreserved according to the method by Lakey J.R.T., Warnock G.L., Kneteman N.M., Ao Z., Rajotte R.V. (1994) "Effects of pre-cryopreservation culture on human islet recovery and in vitro function", Transplant Proc., 26:820 and stored in liquid nitrogen at -196°C. Three days prior to transplantation the cryopreserved islets were rapidly thawed and cultured at 32°C for two days. One day prior to transplantation some of the islets were collected and co-cultured with Sertoli cells for 24 hours.

1           Sertoli cell isolation

          Testes of young S-D rats were removed and  
Sertoli cells were isolated by the method of Cheng C.Y.  
and Bardin C.W. (1987) "Identification of two  
5    testosterone-responsive proteins in Sertoli cell-  
enriched culture medium whose secretion is suppressed by  
cells of the intact seminiferous tubule." J. Biol.  
Chem., 262:12768-12779. Briefly, the testes were  
digested first in DMEM containing 1.0% trypsin, and then  
10   is DMEM containing 1.0% collagenase, type 1, for periods  
of 15 min each, at 37°C. The purified Sertoli cells  
were cultured at 37°C in DMEM/F12 supplemented with  
transferrin, 10 ug/ml, FSH 10 ng/ml, insulin 20 ug/ml  
and 1.0% FCS, for three days. For transplantation,  
15   Sertoli cells and islets were pooled and rats were  
grafted with either a composite consisting of  $5 \times 10^6$   
Sertoli cells and 3,000 islets, or with islets alone (15  
islets/g of body weight).

20           Transplantation of rats

          Female S-D rats, weighing between 170 and 200  
g were made diabetic by means of a single i.v. injection  
of 60 mg/kg of streptozotocin. A total of 31 diabetic  
rats were divided into 3 groups and grafted as follows:  
25   Group 1, a control group (n=8), received a total of 15  
islets/g body weight injected underneath the renal  
capsule. No Sertoli cells were grafted. Following  
transplantation the rats were treated with cyclosporine  
for 55 days: 25 mg/kg for 3 days, 15 mg/kg for 10 days,  
30   10 mg/kg for 10 days and 5 mg/kg for the following 32  
days. Immunosuppression was then stopped. Each rat  
received, in addition, 1-3 U of Ultralente insulin at

1 daily intervals if the 24-hour urine glucose content  
exceeded 1 g. Insulin therapy was stopped on day 55.  
Group 1, a tissue control group (n=8), was given a  
renal, subcapsular injection of a composite of about 5 x  
5 10<sup>6</sup> Sertoli cells and 3,000 islets. No CSA was given.  
Insulin was given as depicted above. Group 3, the  
experimental group (n=15), was transplanted with both  
Sertoli cells and islets and then treated with CSA and  
insulin according to the schedule outlined above.

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#### Posttransplantation evaluation of rats

Plasma glucose levels were obtained at weekly  
intervals. Twenty four hour urine volumes and urine  
glucose contents were recorded daily. A rat was  
15 considered cured of the diabetic process if the  
following criteria applied: A plasma glucose level of  
equal to or less than 10 mmol/L, a 24-hour urine volume  
of less than 15 ml, and immediate reversal to  
hyperglycemia following surgical removal of the grafted  
20 kidney. One normoglycemic rat was mated on day 69 to  
test her ability to become pregnant.

#### Structural analysis of the grafted tissue

Two normoglycemic rats were nephrectomized on  
25 days 117 and 330 and grafted tissue prepared for light  
and electron microscopy. Selawry H.P., Cameron D.F.  
(1992) "Sertoli cell-enriched fractions in successful  
islet cell-transplantation", Cell Trans., 2:123-129.  
Briefly, tissue wedges were immersion-fixed with 5%  
30 glutaraldehyde in 0.1 M collidine buffer for 1 h.,  
washed in buffer, and postfixed for 1 h with 1% osmium  
tetroxide in 0.1 M buffer. Small tissue blocks were cut

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1 from the wedges, and dehydrated through a graded series  
of ethyl alcohols, transferred to propylene oxide, and  
embedded in Epon 812/Araldite plastic resin. Thick (0.5  
5 um) and thin (900 ng) sections were stained routinely  
with toluidine blue and urinal acetate/lead citrate,  
respectively, for structural analysis by light and  
electron microscopy.

The results of the effect of Sertoli cells and  
cyclosporine on survival of xenographic transplantation  
10 of pig islet cells into the renal subcapsular space of  
diabetic female rats are shown in Table 7.

TABLE 7

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Group (n)	Sertoli Cells	CsA	Graft Survival (days)
1 (8)	-	+	0,0,0,0,0,0,0,0
2 (8)	+	-	0,0,0,0,0,0,0,0
3 (15)	+	+	0,0,0,0,0, 71, 77, 96, 117*, 148#, >154, >165, >327, 330*

20 \* rats nephrectomized to remove the xenograft

# rat died during a cardiac puncture

As shown in Table 7, none of the rats grafted with  
islets alone and then given CsA and low-dose insulin  
25 (Group 1) became significantly less hyperglycemic.  
Further, none of the rats grafted with a composite of  
islets and Sertoli cells, but without CsA, showed any  
improvement of hyperglycemia (Group 2). Of 15 rats  
grafted with islets and Sertoli cells and then given CsA  
30 (Group 3), 10 showed evidence of reversal of the  
diabetic state. Four of the ten are still normoglycemic  
for periods of more than 154, 165, 165, and 327 days,

1 respectively. The normoglycemic rats who were  
nephrectomized on days 117 and 330, became hyperglycemic  
immediately. Their plasma glucose levels were 4.9  
mmol/L, and 8.2 mmol/L, prior to, and 20.7 mmol/L, and  
5 32.2 mmol/L, respectively, following nephrectomy. A  
female rat who was mated on day 69 became pregnant and  
delivered a total of 10 pups on day 89, all of whom she  
nursed successfully while remaining normoglycemic. She  
died on day 148 as a result of a cardiac puncture.  
10 Three of 10 rats regressed into hyperglycemia on days  
71, 77, and 96, respectively, after a short period of  
euglycemia.

These results demonstrate that prolonged survival  
of a discordant islet xenograft (pig to rat) can be  
15 achieved in female diabetic rats. Survival of islet  
xenografts depended upon two factors which had to be  
administered concomitantly: Co-transplantation with  
Sertoli cells and treatment with cyclosporine.

The response of total urine volumes following  
20 transplantation with a composite of pig islet and rat  
Sertoli cells measured at 10-day intervals over an 80  
day period for 7 of the improved rats showed an average  
daily urine volume of  $27.0 \pm 13.0$  ml/rat during the  
first 10-day period, which slowly declined to a mean of  
25  $12.0 \pm 4.0$  ml/rat, 70 days following transplantation.

Tissue morphology studies shown in Figure 10 show  
that the tissue and cellular structure of kidney  
parenchyma appeared normal in the rat nephrectomized 117  
days following transplantation. Normal appearing islets  
30 with structurally distinct B-cells were visible in well  
vascularized areas subjacent to the kidney capsule.  
Additionally, normal appearing Sertoli cells were



1 observed adjacent to the transplanted islets along with  
numerous lymphocytes. No plasma cells were identified  
at the transplantation site. Viable endocrine cells  
5 were similarly observed in the subcapsular renal space  
of the rat nephrectomized 330 days following  
transplantation.

These studies show that significant prolongation of  
survival of a discordant islet xenograft can be achieved  
without sustained immunosuppression. These studies  
10 demonstrate that the mechanism by which Sertoli cells  
promote islet xenograft survival is three-fold: (1)  
Sertoli cells stimulate the recovery of islets damaged  
during transplantation (i.e. improve the yield and  
function of cultured islets), (2) Sertoli cells protect  
15 grafted islets from immunologic rejection by producing  
factors which strongly suppress proliferation of T-  
cells, and (3) Sertoli cells protect grafted islets from  
the toxic effects of cyclosporine.

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EXAMPLE 7

This study shows a method of isolating and cryopreserving porcine pancreatic islets for future xenographic transplants in mammals.

Male piglets, < 7 days old and weighing 2+ kg were used as donors. The pancreases, weighing  $1.4 \pm 0.3$  g, were harvested and injected with DMEM solution containing 2 mg/ml collagenase XI. The distended pancreas was incubated in a shaking water bath at 39°C for 17 min. The digested tissue was filtered through a 500  $\mu$ m stainless steel filter and filtrates were washed x 3 with cold DMEM. Without further purification the cells were cultured in M199 and 10% horse serum at 32°C for 7 days. The islet cells were then cryopreserved using standard procedures. At specified intervals islets were thawed and cultured in M199, both in presence, and isolated from testes of male piglets according to a standard method.

To test functional capacity, islets cultured for 3 and 7 days were assessed for insulin release in static incubation. In separate experiments, effect of insulin secretagogues was tested on islets cultured with and without Sertoli cells. The results of this study are shown in Tables 8 and 9.

**TABLE 8**

Effect of insulin secretagogues, glucose and glucose plus Forskolin, on Insulin release from Incubated and Frozen/thawed (F/T) islets in the presence and absence of Pig Sertoli cells.

	Insulin Release (uU/ml/10islets)		
	3.3mmol/L glucose	16.7 mmol/L glucose	16.7 mmol/L glucose + 100 umol Forskolin
Day 3 Incubated with Sertoli cells	42.3 ± 1.2	112.8 ± 17.7*#	267.7 ± 43.0**#
Day 3 Incubated alone	31.3 ± 2.1	57.3 ± 3.8*	123.4 ± 15.3**
Day 7 Incubated with Sertoli cells	22.9 ± 1.9	64.5 ± 6.4*#	153.9 ± 14.6**
Day 7 Incubated alone	21.3 ± 1.2	37.3 ± 6.0*	120.3 ± 11.4**
Day 3 F/T with Sertoli cells	20.6 ± 4.3	44.9 ± 9.9*	77.1 ± 13.7**
Day 3 F/T alone	11.7 ± 2.3	27.9 ± 6.6*	54.5 ± 10.7**

Anova Test: \* vs 3.3mmol/L p 0.05, \*\* vs both 3.3 & 16.7 mmol/L P<0.05 # with sertoli cells vs islets alone P<0.05

**TABLE 9**

Effect of Sertoli cells on insulin content of incubated and frozen-thawed piglet islets.

	Insulin content (uU/10 islet(s))	
	Islets alone	Islets & Sertoli cells
Incubated D1	257.0 ± 19.6	391.1 ± 51.4 <sup>-</sup>
Incubated D3	201.1 ± 19.1#	400.1 ± 41.0 <sup>-</sup> #
Incubated D7	179.1 ± 26.2#	271.9 ± 39.9 <sup>-</sup> #
Frozen D3/Thaw D3	52.4 ± 10.3	132.5 ± 35.1
Frozen D7/Thaw D3	10.4 ± 0.9	35.1 ± 6.2

Anova islets + Sertoli cell vs. islet alone P<0.05  
# Incubated islets D3, D7 vs. Frozen D3, D7 P<0.05

These results show that: (1) large numbers of neonatal porcine islets can be isolated by a simple method; (2)

1 cryopreservation and thawing results in about 40% loss  
in number of islets in the absence of Sertoli cells and  
about a 20% loss in the presence of Sertoli cells ; (3)  
5 cultured islets have the ability to respond to both  
glucose and glucose + Forskolin as insulin  
secretagogues; (4) the functional capacity of the co-  
cultured islet was enhanced two-fold in the presence of  
Sertoli cells; (5) following cryopreservation and  
10 thawing, islets recover more rapidly in presence of  
Sertoli cells and the response to both glucose and  
glucose + Forskolin was enhanced two fold in the  
presence of Sertoli cells.

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1 I claim:

✓ 1. A method of treating a disease that results from a deficiency of a biological factor in a mammal wherein said method comprises administering Sertoli cells and a therapeutically effective amount of cells that produce said biological factor to a mammal in need of such treatment, wherein said Sertoli cells are administered in an amount effective to create an immunologically privileged site.

10 2. The method of Claim 1 wherein said mammal is a human.

3. The method of Claim 1 wherein said biological factor is a hormone.

15 4. The method of Claim 1 wherein said biological factor is insulin and said disease is diabetes mellitus.

5. The method of Claim 4 wherein said cells that produce said biological factor are pancreatic islet of Langerhans cells.

20 6. The method of Claim 1 wherein said cells that produce said biological factor are cells transformed by a nucleic acid encoding said biological factor.

25 7. The method of Claim 1 wherein said administering is by transplantation.

8. The method of Claim 1 wherein said Sertoli cells are administered in a dosage ranging from  $10^5$  to  $10^{10}$  cells.

30 9. The method of Claim 1 wherein said cells that produce said biological factor are administered in a dosage of from  $10^5$  to  $10^{10}$  cells.

1           10. The method of Claim 7 wherein said  
transplantation is by xenograft.

          11. The method of Claim 7 wherein said  
transplantation is by allograft.

5           12. The method of Claim 1 which further  
comprises administering an immunosuppressive agent.

          13. The method of Claim 12 wherein said  
immunosuppressive agent is administered for a time  
sufficient to permit said transplanted cells to be  
10 functional.

          14. The method of Claim 12 wherein said  
immunosuppressive agent is cyclosporine.

          15. The method of Claim 14 wherein said  
cyclosporine is administered at a dosage of from 5 to 40  
15 mg/kg body wt.

          16. The method of Claim 1 which further  
comprises administering a therapeutically effective  
amount of exogenous biological factor following the  
transplantation of said cells that produce said  
20 biological factor.

          17. The method of Claim 1 wherein said cells  
that produce said biological factor are co-cultured with  
Sertoli cells in tissue culture.

          18. The method of Claim 17 wherein said cells  
that produce said biological factor are cryopreserved  
prior to co-culturing with Sertoli cells in tissue  
25 culture.

          19. A method of treating diabetes mellitus in  
a mammal wherein said method comprises administering to  
30 a diabetic mammal Sertoli cells in an amount effective  
to create an immunologically privileged site and a

1 therapeutically effective amount of pancreatic islet of Langerhans cells.

20. The method of Claim 19 wherein said diabetes mellitus is type I or type II.

5 21. The method of Claim 19 wherein said mammal is a human.

22. The method of Claim 19 wherein said Sertoli cells are human, bovine or porcine.

10 23. The method of Claim 19 wherein said pancreatic islet of Langerhans cells are human, bovine or porcine.

24. The method of Claim 19 wherein said administering is by transplantation.

15 25. The method of Claim 24 wherein said transplantation is by injection into the renal subcapsular space.

26. The method of Claim 24 wherein said transplantation is by injection into the subcutaneous facie.

20 27. The method of Claim 19 wherein said Sertoli cells are administered at a dosage ranging from  $10^5$  to  $10^{10}$  cells.

25 28. The method of Claim 19 wherein said islet of Langerhans cells are administered at a dosage ranging from 5-1000 islet cells/g body wt.

29. The method of Claim 19 which further comprises the administration of an immunosuppressive agent.

30 30. The method of Claim 29 wherein said immunosuppressive agent is administered for a time sufficient to permit the transplanted islets to be functional.

1           31. The method of Claim 29 wherein said  
immunosuppressive agent is cyclosporine.

5           32. The method of Claim 31 wherein said  
cyclosporine is administered at a dosage of 5 to 40  
mg/kg body wt.

33. The method of Claim 19 which further  
comprises administering a therapeutically effective  
amount of insulin following transplantation of said  
pancreatic islet of Langerhans cells.

10           ✓ 34. A method of creating an immunologically  
privileged site in a mammal wherein said method  
comprises transplanting isolated Sertoli cells into a  
mammal.

15           35. The method of Claim 34 wherein said mammal  
is a human.

36. The method of Claim 34 wherein said  
Sertoli cells are injected into the renal subcapsular  
space.

20           37. The method of Claim 34 wherein said  
Sertoli cells are injected into the subcutaneous facie.

38. The method of Claim 34 wherein said  
Sertoli cells are transplanted at a dosage ranging from  
 $10^5$  to  $10^{10}$  cells.

25           39. The method of Claim 34 wherein said  
Sertoli cells are human, bovine or porcine.

30           ✓ 40. A method of enhancing the recovery and  
proliferation of ex vivo cells comprising co-culturing  
said cells with Sertoli cells for a time and under  
conditions sufficient to achieve said enhanced recovery  
and proliferation.



1       ✓ 41. A pharmaceutical composition comprising  
Sertoli cells and cells that produce a biological factor  
and a pharmaceutically acceptable carrier.

5       42. The composition of Claim 41 wherein said  
biological factor is a hormone.

43. The composition of Claim 41 wherein said  
cells that produce a biological factor are pancreatic  
islet of Langerhans cells.

10       44. The composition of Claim 41 wherein said  
cells that produce said biological factor are cells that  
are transformed by a nucleic acid encoding said  
biological factor.

15       ✓ 45. A pharmaceutical composition comprising  
Sertoli cells, pancreatic islet of Langerhans cells and  
a pharmaceutically acceptable carrier.

✓ 46. A pharmaceutical composition comprising  
Sertoli cells and a pharmaceutically acceptable carrier.

20       ✓ 47. A compartmentalized kit adapted to receive  
a first container adapted to contain Sertoli cells and a  
second container adapted to contain cells that produce a  
biological factor that is absent or defective in a  
disease.

25       ✓ 48. A compartmentalized kit adapted to receive  
a first container adapted to contain Sertoli cells and a  
second container adapted to contain pancreatic islet of  
Langerhans cells.

30       ✓ 49. An article of manufacture comprising a  
packaging material and Sertoli cells contained within  
said packaging material, wherein said Sertoli cells are  
effective for creating an immunologically privileged  
site in a mammal, and wherein said packaging material  
contains a label that indicates that said Sertoli cells

1 can be used for creating an immunologically privileged  
site in a mammal.

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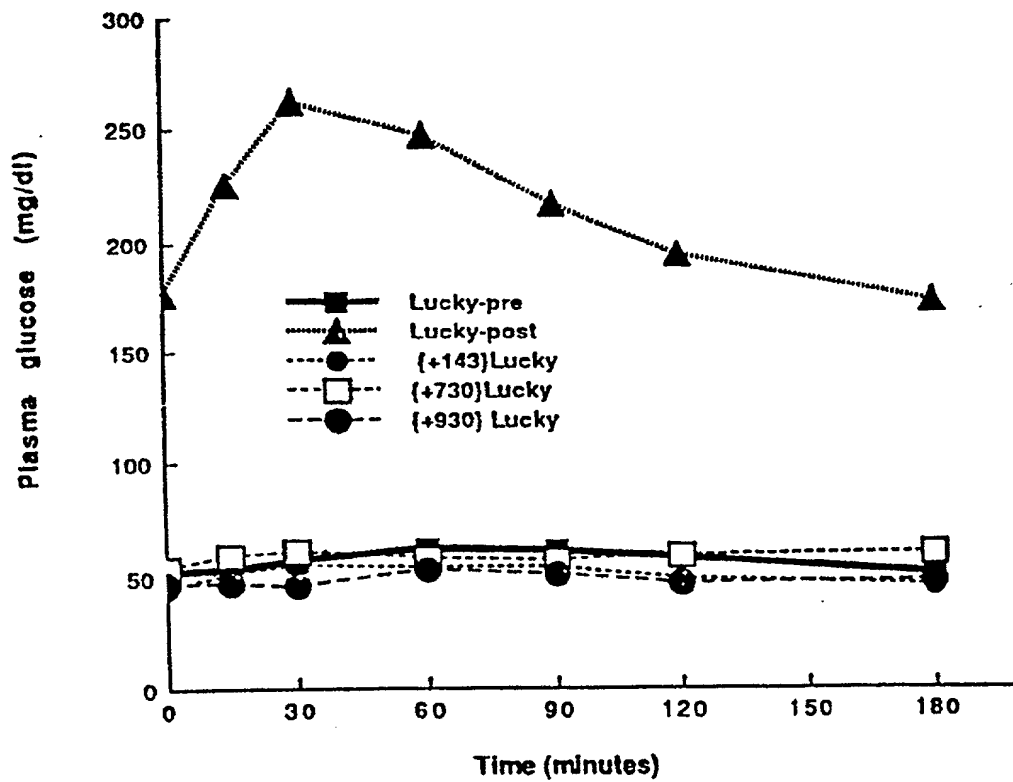


FIGURE 1

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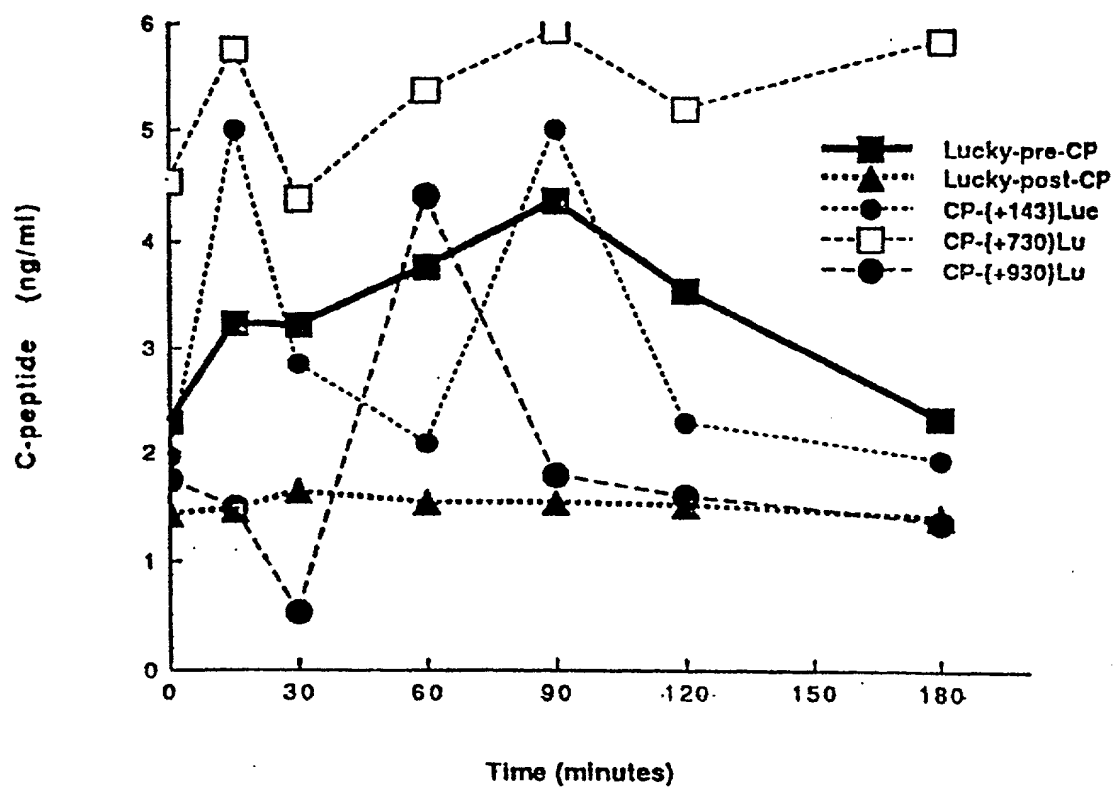


FIGURE 2

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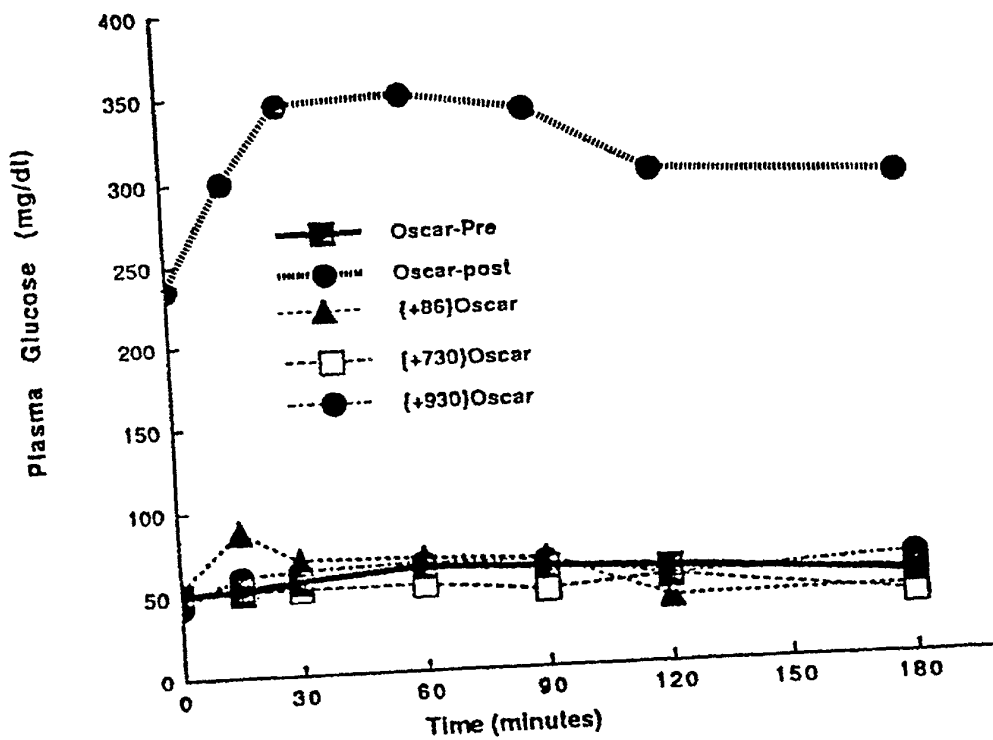


FIGURE 3

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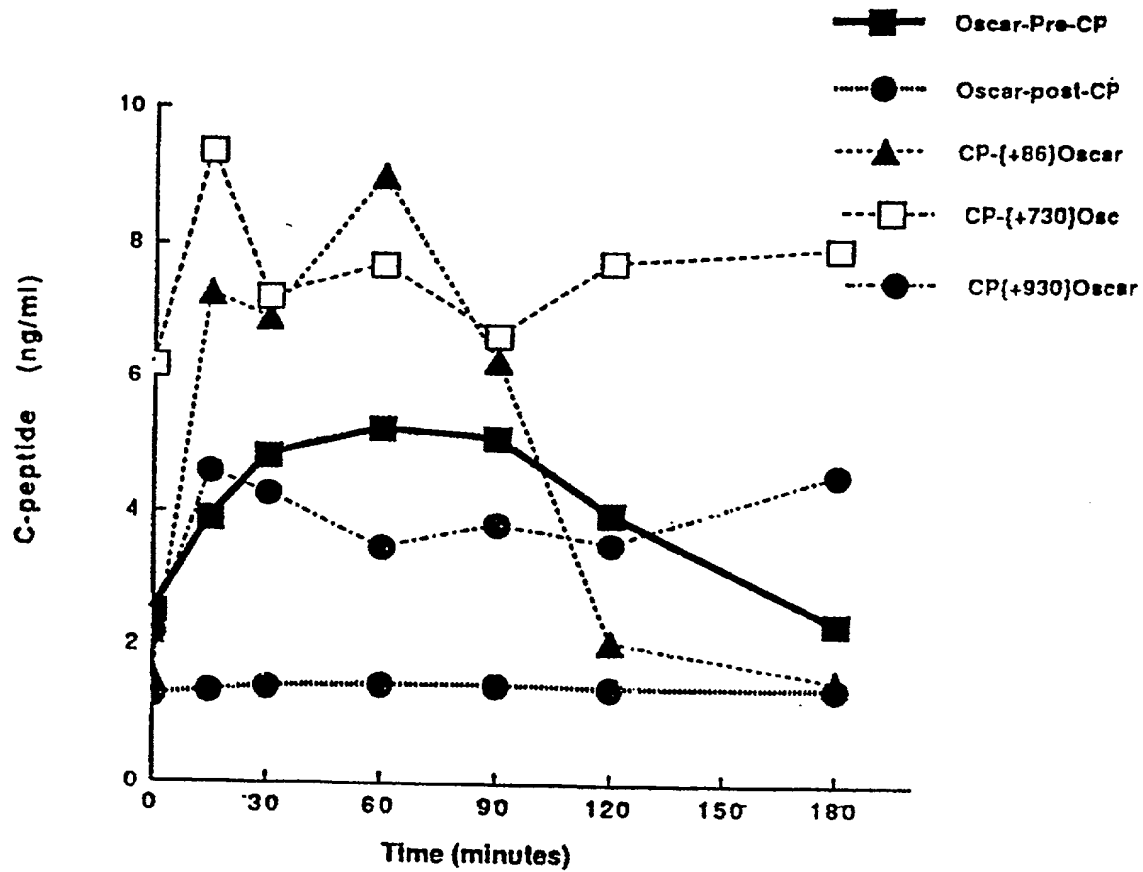
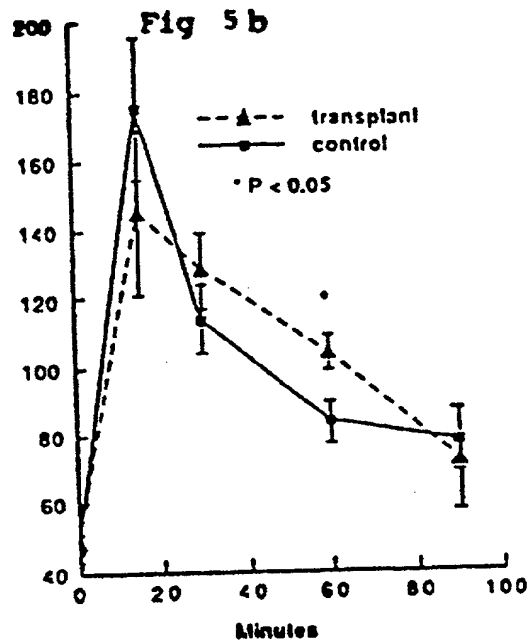
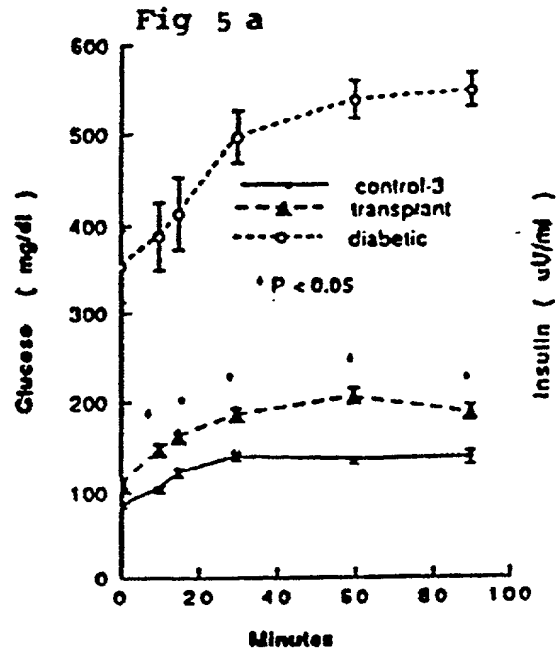


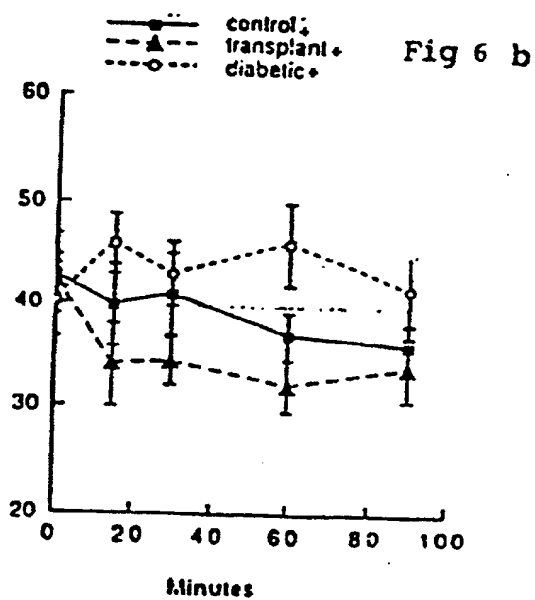
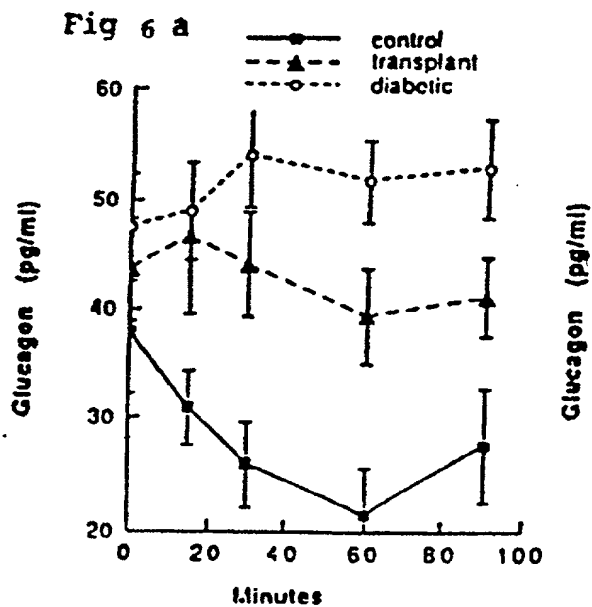
FIGURE 4

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FIGURES 5a and 5b

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**FIGURES 6a and 6b**



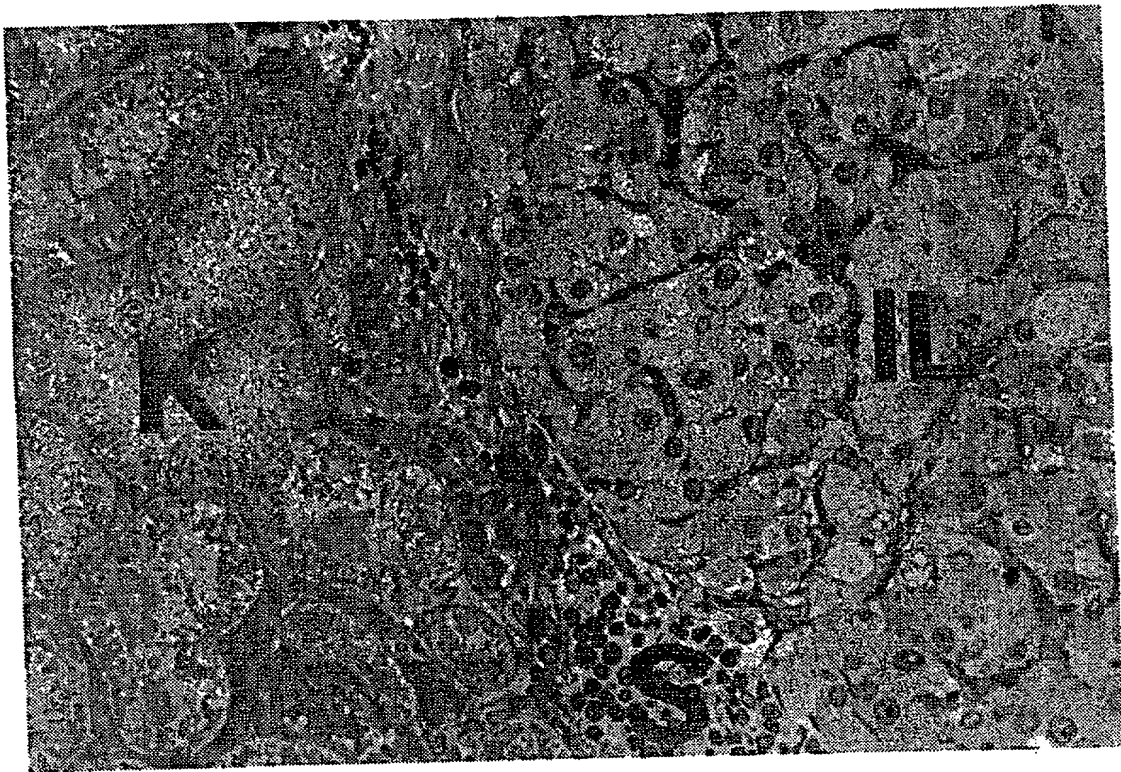


FIG. 7

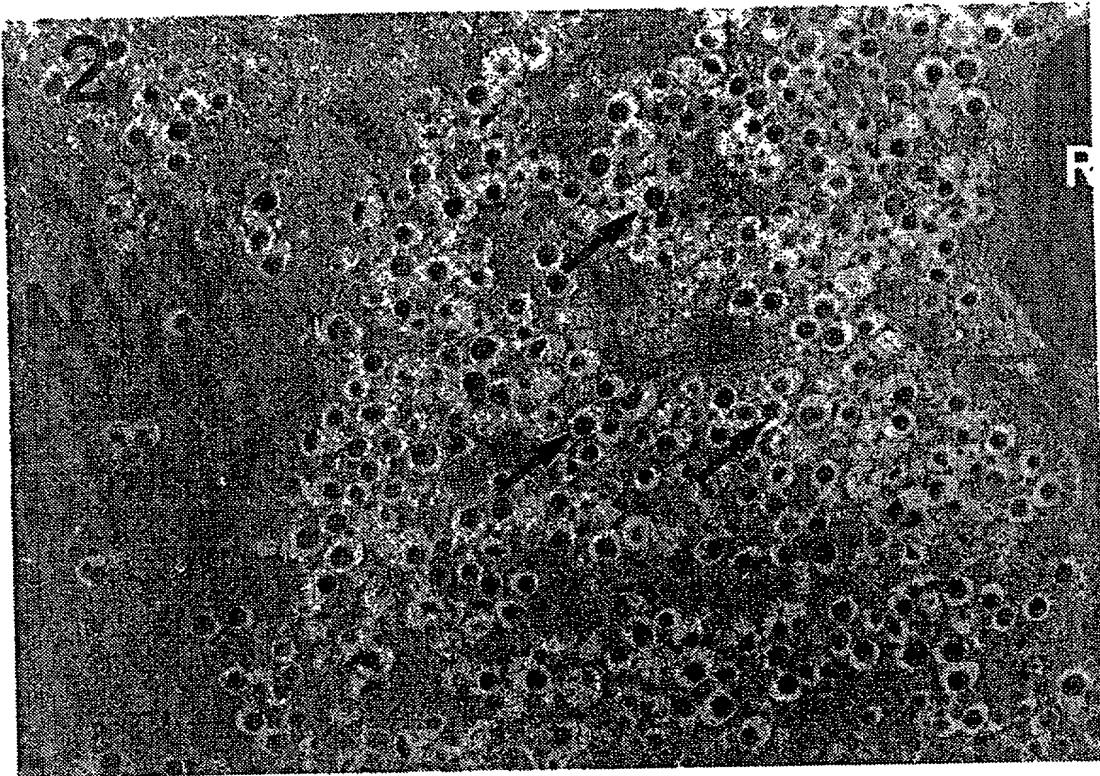


FIG. 8

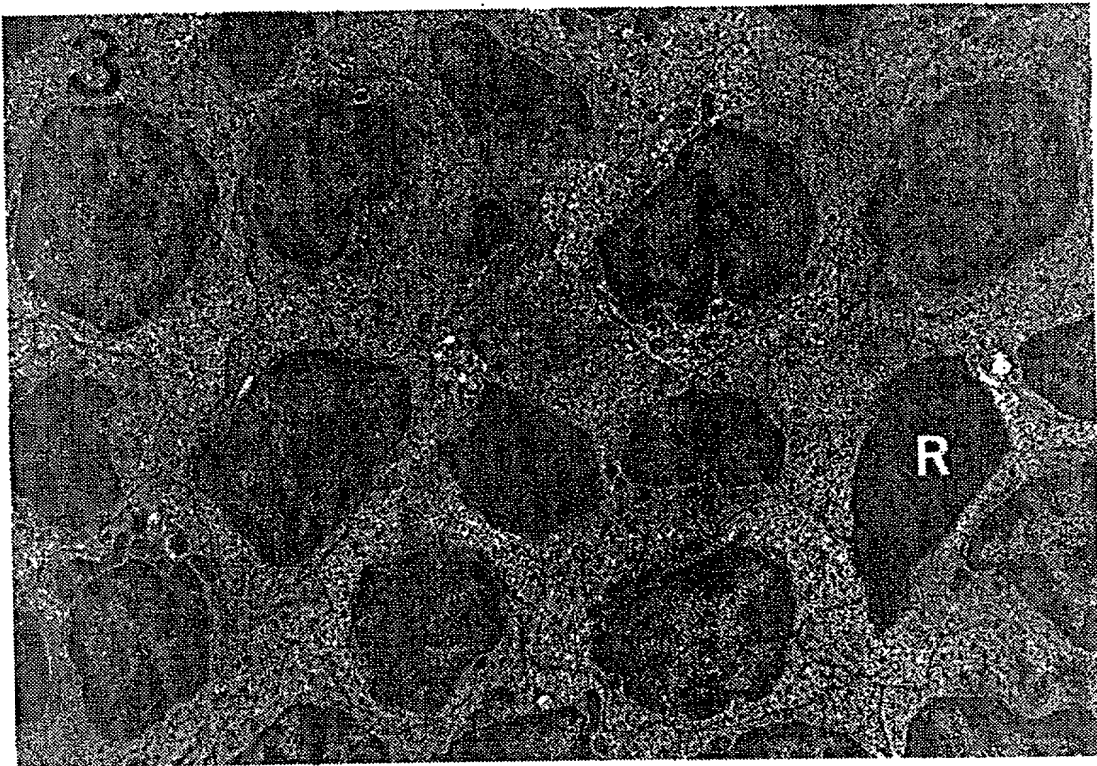


FIG. 9

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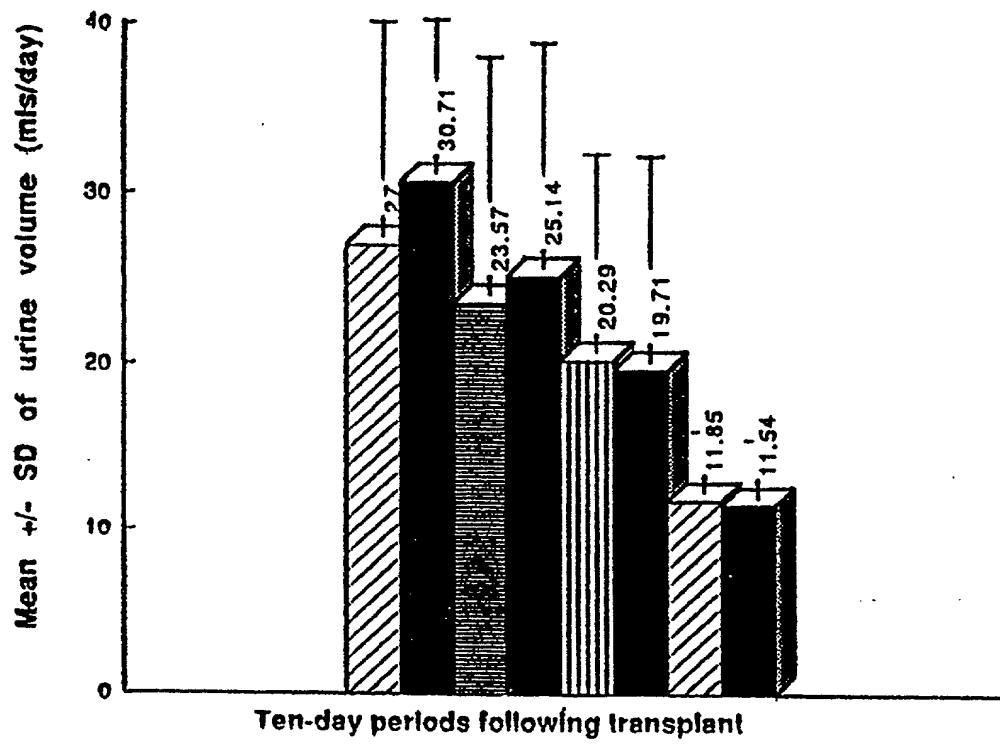


FIGURE 10

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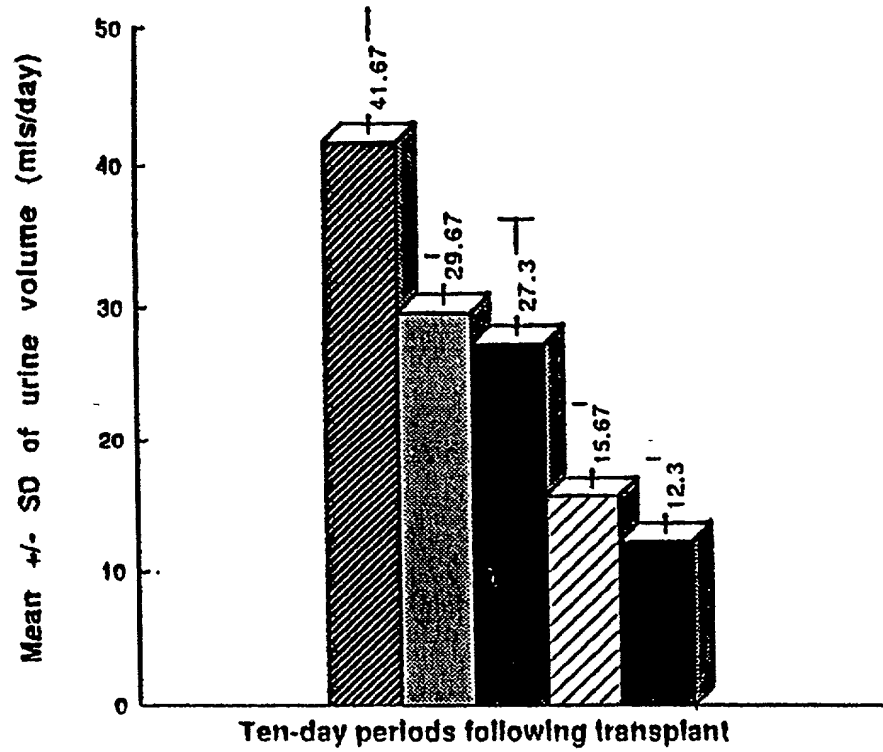


FIGURE 11

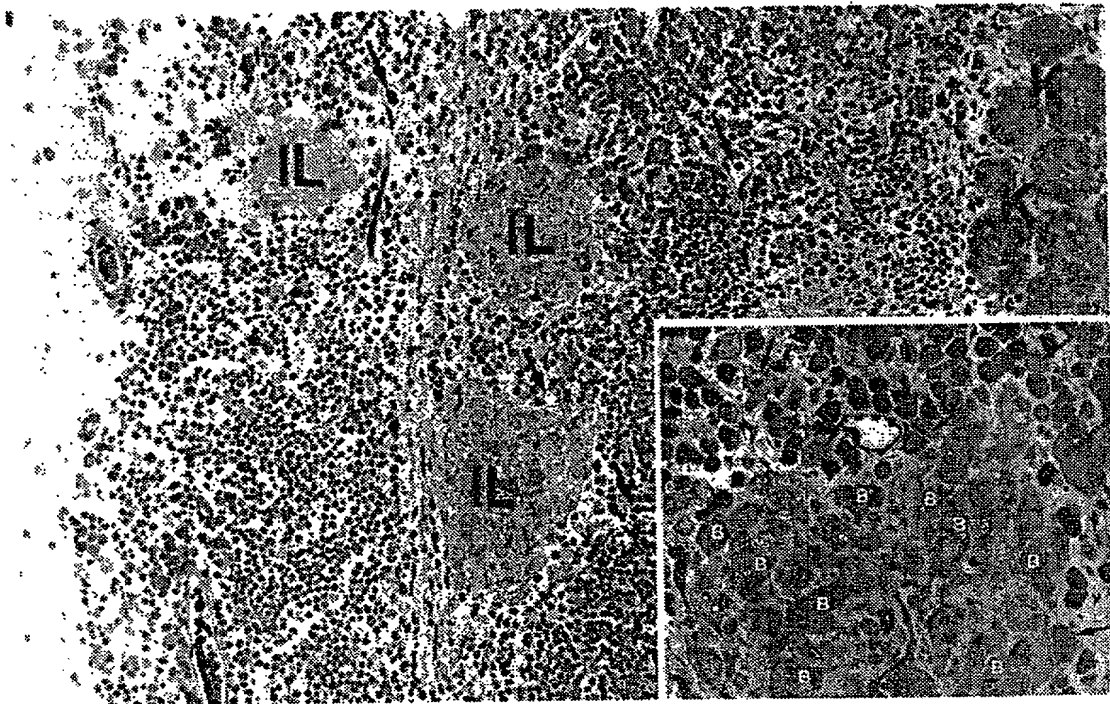


FIG. 12



06/03/97

DECLARATION  
AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. underneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

## METHODS OF TREATING DISEASE USING SERTOLI CELLS AND ALLOGRAFTS OR XENOGRAFTS

and for which a patent application:

- ☐ is attached hereto and includes amendment(s) filed on \_\_\_\_\_ (if applicable)
- ☒ was filed in the United States on November 8, 1996 as Application Serial No. 08/747,122 (for declaration not accompanying application)
- with amendment(s) filed on \_\_\_\_\_ (if applicable)
- ☐ was filed as PCT international application Serial No. \_\_\_\_\_ on \_\_\_\_\_ and was amended under PCT Article 19 on \_\_\_\_\_ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES <input type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED
08/211,695	April 13, 1994		X	

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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DATE	DATE <u>5/9/97</u>	DATE
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DATE	DATE	DATE